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Abstract

Amoebae from the genus *Acanthamoeba* are facultative pathogens of humans and other animals. In humans they most frequently infect the eye causing a sight threatening infection known as *Acanthamoeba* keratitis (AK), and also cause an often fatal encephalitis (GAE). A mannose-binding protein (MBP) has been identified as being important for *Acanthamoeba* infection especially in AK. This lectin has previously been characterized from *Acanthamoeba castellanii* as consisting of multiple 130 kDa subunits. MBP expression correlates with pathogenic potential and is expressed in a number of *Acanthamoeba* species. Here we report the purification of a similar lectin from *Acanthamoeba culbertsoni* and the production of a monoclonal antibody to it. The *A. culbertsoni* MBP was isolated by affinity chromatography using α-D-mannose agarose and has an apparent molecular weight of 83 kDa. The monoclonal antibody is an IgM that is useful in both western blots and immunofluorescence. We expect that this antibody will be useful in the study of the pathology of *A. culbertsoni* and in its identification in clinical samples.

Key words: *Acanthamoeba culbertsoni*; mannose-binding protein; monoclonal antibody; detection

1. Introduction

Amoebae from the genus *Acanthamoeba* are the causative agents of *Acanthamoeba* keratitis (AK), a sight threatening eye infection, and granulomatous amoebic encephalitis (GAE), a condition that predominantly occurs in immunocompromised individuals and which is typically fatal (Schuster and Visvesvara, 2004; Walochnik et al., 2008). In GAE, the skin and occasionally the olfactory neuroepithelium are thought to act as portals of entry, and inflammation may be observed at these primary foci. Descriptions of approximately 150 cases of GAE caused by *Acanthamoeba* have been published worldwide, and less than 10 of these patients have survived (Schuster and Visvesvara, 2004; Walochnik et al., 2008). *A. culbertsoni* causes GAE (Martinez, 1991) by triggering cell necrosis, as it causes cytopathic effects on target cell, such as contraction, vesiculation and nuclear condensation (Shin et al., 2001). *Acanthamoeba* trophozoites destroy nerve cells by contact-dependent cytolysis and also by the ingestion of nerve cells through amoebastomes (Pettit et al., 1996).

During the process of AK it is reported that direct contact between the amoebae and the cornea is important in the development of the full pathogenic process (Cao et al, 1998; Gonzalez-Robles et al, 2006) and the interaction with the host cell is mediated through surface carbohydrates. Some of the proteins involved have been identified (Shin and Im, 1992; Lee et al., 2007; Kim et al., 2012), but most importantly, a mannose-binding protein (MBP) has been identified as mediating host cell adhesion with *Acanthamoeba* (Garate et al., 2004; Kim et al., 2012; Yoo and Jung, 2012; Khan, 2007). Incubation of amoebae with mannose resulted in inhibition of adhesion on both collagen and laminin (Rocha-Azevedo et al., 2010) and immunization of hamsters against MBP is protective against AK (Garate et al, 2006)

demonstrating the importance of MBP in the pathology.

Antibodies against *Naegleria fowleri* antigens, another pathogenic free-living amoeba have proven useful to diagnose infections in experimental animals (Kollars and Wilhelm, 1996; Lee, 2007; Ryu & Im, 1992). In this study, a monoclonal antibody to *A. culbertsoni* MBP was produced to aid the study of the protein's function in infection. This antibody was used in immunocytochemistry to localize MBP and may be useful in diagnosis of GAE.

2. Materials and methods

2.1. Culture of A. culbertsoni trophozoites and purification of its MBP

A. culbertsoni trophozoites (ATCC NO. 30171; Kim et al, 1988; Kong et al,1993) were grown without shaking in 12 ml of PYG medium (proteose peptone 0.75% (w/v) (Kisan Bio, Seoul, Korea), yeast extract 0.75% (w/v) (Kisan Bio, Seoul, Korea) and glucose 1.5% (w/v) (Sigma-Aldrich Co., St. Louis, MO, USA)) in a 75T culture flask at 25□. For the cross-reactivity, *A. castellanii* (ATCC NO. 50492), *A. polyphaga* and *A. astronyxis* kindly provided by Prof. Shin at Ajou University, South Korea were cultured by the PYG medium. For the purification of *A. culbertsoni* trophozoites, *A. culbertsoni* trophozoites were washed with phosphate buffered saline (PBS) (Sigma-Aldrich Co., St. Louis, MO, USA) three times, and after centrifugation, was then lysed with a lysis buffer (50 mM Tris-HCl, 50 mM $CaCl_2$, 150 mM $NaCl_2$, 1 mM phenylmethane sulfonyl fluoride (PMSF), 2 mM β-mercaptoethanol, 0.5% CHAPS) (Sigma-Aldrich Co., St. Louis, MO, USA) using a sonicator (20 W, total 2 min) (Garate *et al*., 2004). The amoeba lysates were purified by centrifugation (13,000 rpm, 1 h, 4□)

and were chromatographed on an α-D-mannose agarose (Sigma-Aldrich Co., St. Louis, MO, USA) affinity column (Qiagen, CA, USA). The unbound components were removed by washing buffer with the elution buffer and bound components were eluted by 1 ml of 150 mM mannose (Kim et al., 2012, Garate et al., 2005).

2.2. Immunization of the MBP to BALB/c mice

BALB/c (NARA Biotech, Seoul, Korea) mice were injected intra-abdominally with 50 μg of antigen mixed with 100 μl of Freund's complete adjuvant (Sigma-Aldrich Co., St. Louis, MO, USA) at weekly intervals for a period of 2 weeks (Seong, 2016). At the two weeks after the 5^{th} immunization, sera from the tail of the mice were taken and measured for antibody titers. After the mice antibody titer was confirmed increased, 25 ng of MBP antigen was injected intravenously into the mice tail for boosting. After four days, the mice spleens were extracted and were used in cell fusion. All experiments to use animals were approved by Namseoul Animal Committee (NSU-16-04).

2.3. Production of monoclonal antibodies against the MBP

The cell fusion technique used was a slight modification of the methods of Lee and Kim (Kim et al., 2012; Lee et al., 2007). Briefly, BALB/c mice spleen cells were mixed with a RBC lysis buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM EDTA, pH 7.5) in order to remove red blood cells for 5 min at RT, and were prepared for cell fusion with myeloma cells. Myeloma F0 cell (ATCC No. PTA-11450) was grown in complete DMEM of 10% FBS (Welgene,

Gyeongsan, Korea). The myeloma cells and the spleen cells above were suspended together in incomplete DMEM by adding polyethylene glycol (PEG) solution. The mixture was cultured in 96-well plate with hypoxanthine-aminopterin-thymidine (HAT, Sigma) medium at 37□, in a 5% CO_2 incubator for 3 days. Then culture medium was replaced with 200 μl of hypoxanthine-thymidine (HT) medium. The hybridoma cells were observed with an inverted microscope after cell fusion and antibodies titers were evaluated by ELISA. The hybridoma cells producing antibodies were scaled up in 24-well plates and consequently 75T culture flask (Nunc, USA) by culturing at 37□, in a 5% CO_2 incubator. Hybridoma cells producing monoclonal antibody were selected by the limiting dilution method (Kim et al., 2012). Hybridoma cells were seeded on a 96-well culture plate with 0.25 cells per well. After incubation about ten days, the hybridoma cells were checked with an inverted microscope and then screened by ELISA. Colonies showing a high antibody by ELISA were transferred to 24-well culture plate and then moved into 75T culture flask. Large scale production of monoclonal antibodies was obtained from ascites of mice injected with colonies.

2.4. Characterization of a monoclonal antibody

The monoclonal antibody isotypes were determined by using the IsoStrip mouse monoclonal antibody isotyping kits (Roche, CA, USA) according to the manufacturer's instructions. All isotypes of Immunoglobulin A, Immunoglobulin M, Immunoglobulin G2a and Immunoglobulin G2b, and Immunoglobulin G3 were checked.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot

In order to analyze the purified MBP and evaluate the monoclonal antibody to react the purified MBP, SDS-PAGE and western blot were performed as described by Laemmli (Laemmli, 1970; Seong et al., 2017). The samples were mixed SDS-PAGE buffer containing 2-mercaptoethanol, boiled for 5 min, and loaded on to 12% polyacrylamide gels. The purified MBP were migrated in SDS-PAGE gels, and the protein bands were stained with silver staining solution (Samchun, Pyeongtaek, Korea) for 3 h. The MBP was transferred to nitrocellulose membranes (GE Healthcare life sciences, Buckinghamshire, UK) at 250 mA for one and half an hour in transfer buffer (25 mM sodium phosphate, pH 7.5). The nitrocellulose membranes were blocked with PBS containing 3% bovine serum albumin (BSA) at room temperature (RT) overnight, and washed three times with PBS including with 0.05% Tween 20 (PBST). The nitrocellulose membranes were reacted with culture fluid of cell for overnight at RT. After reaction with the monoclonal antibody, the nitrocellulose membranes were washed three times with PBST and incubated for 2 h at RT with alkaline phosphatase-conjugated goat anti mouse immunoglobulin G (IgG) (Sigma-Aldrich Co., St. Louis, MO, USA) as a secondary antibody. After three times wash with PBST, the reaction was developed following incubation at RT with 5-bromo-4-chloro-3-indolyl-1-phosphate and nitroblue tetrazolium chloride (BCIP/NBT) (Sigma-Aldrich Co., St. Louis, MO, USA) (Lee et al., 2007).

2.6. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed to evaluate the polyclonal sera and the monoclonal antibodies from hybridomas. Briefly, the purified MBP at 1 μg/ml mixed with coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) was coated onto 96-well

ELISA plates and then blocked with 3% bovine serum albumin (BSA) (Sigma-Aldrich Co., St. Louis, MO, USA) in PBS for 37□ for 1 h. After the plates were washed three times with PBST, each serum was diluted to 1:200 in PBS (pH 7.2). In particular, for the hybridomas, 100 μl of culture supernatants in 96-well culture plates was added. After the incubation at 37□ for 2 h, the wells were washed three times with PBST, and 100 μl of goat anti-mouse IgG conjugated alkaline phosphatase (Sigma-Aldrich Co., St. Louis, MO, USA) diluted 1:10,000 in PBS were added to the wells. Final reaction was developed with 4-nitrophenyl phosphate disodium salt hexahydrate tablet (*p*-NPP) (Sigma-Aldrich Co., St. Louis, MO, USA) under wavelength of 405 nm.

2.7. Immunocytochemistry for localization of the MBP

To observe the localization of the MBP in trophozoites, immunocytochemistry was performed. The trophozoites were cultured at 6-well cell culture plate overnight. After the culture medium was discarded, the trophozoites were washed with 0.82% saline three times. 200 μl of 10% formalin in 0.85% saline was added and the plate was incubated at RT for 50 min. The trophozoites were washed with 0.82% saline three times, added 200 μl of 1% NH_4OH to render them permeable, and then incubated at RT for 20 min. The following washing steps were same above. After blocking with 3% BSA in 0.85% saline, the cells were incubated with monoclonal antibody at RT overnight. After several washings, the amoebae were reacted with secondary antibody of an AffiniPure anti-mouse IgG (whole molecule)-fluorescein isothiocyanate (FITC) antibody produced in goat (Sigma-Aldrich Co., St. Louis, MO, USA) (1:200 dilution with 3% BSA) at RT for 2 h and washed with PBST. The trophozoites were

analyzed under a fluorescent microscope using standard FITC filters (Motic BA & Moticam pro 252A, GR, HK).

3. Results

3.1. Purification of MBP by a saccharide of methyl-alpha-D-mannopyranoside

We have purified a MBP from *A. culbertsoni* trophozoites using a methyl-alpha-D-mannopyranoside immobilized column. The purified MBP has an approximate 83 kDa MW (Fig. 1) in close agreement with the predicted molecular weight of the *A. castellanii* gene (Garate et al, 2004).

3.2. Antisera titer by the immunization of MBP

MBP immunization was performed five times to BALB/c mice and their sera were isolated from their tails. The polyclonal antibody titer was confirmed in the mice serum by ELISA. Among 10 mice, only three mice were chosen due to high titer. The titer ranged between 0.277 and 0.598, which was reflected with absorbance at 405 nm (Fig. 2). The mouse showing the highest titer (0.598) was used as the source of hybridoma cells.

3.3. Selection of a clone showing high affinity

All clones showing group formation were grown in a 96-well culture plate, and the selected

hybridoma clones were transferred into a 75T flask culture flask. A hybridoma, 2BA9 of higher titer was selected and its absorbance of the 2BA9 clones from a 75T flask culture flask was increased to 3.968 (Fig. 3A). The 2BA9 clones were transferred into 96-well plates and then were limit-diluted. Finally, DG11 of a limit diluted-monoclonal antibody was selected and its absorbance was measured with 2.505 in a 96-well culture plate and 4.000 in a 75T flask (Fig. 3B).

3.4. Isotyping of a monoclonal antibody and antigenicity of the DG11

The monoclonal antibody of DG11 was analyzed for isotyping by a mouse monoclonal antibody isotyping kit (Roche, BS, EU). It represented a IgM class of kappa chain (data not shown). To observe whether DG11 of a monoclonal antibody reacted with the purified MBP of about 83 kDa, western blot was performed. As with the data of silver staining, the DG11 was reacted with a purified MBP band (Fig. 4).

3.5. Localization of MBP by immunocytochemistry

In order to observe the localization of the MBP, immunocytochemistry was performed by polyclonal serum and DG11 monoclonal antibody (Fig. 5). The MBP by polyclonal antibody and DG11 was shown widely distributed in cytoplasm and cell membranes. In particular, as shown at DG11, when the amoeba moved forward, the MBP was concentrated in the direction of its movement (Fig. 5). For the cross-reactivity, A. *castellanii, A. polyphaga* and *A. astronyxis* did not show fluorescence by DG11 above.

4. Discussion

A. culbertsoni can cause chronic GAE in experimental animals (Hyun *et al*, 1992) and humans (Martinez, 1991). Its pathophysiology is not well known yet. However, a contact-dependent pathway via saccharides or proteins has been of interest. The contact-dependent pathway can sometimes accompany contact-independent pathway, e.g., serine protease, cysteine protease, etc. For instance, *Acanthamoeba*-conditioned media exhibited similar effects indicating *Acanthamoeba*-mediated blood-brain barrier permeability is contact-independent (Alsam et al., 2005), etion of proteinases from parasite has a direct relation with the cause of infiltration and infection to a host, and also has an effect on the defense mechanism of a parasite against a host, nutrition supply, and life cycle (North, 1982). Moreover, one of molecules associated with the contact-dependent pathway was the mannose-binding protein (MBP) which played an important role in the contact-dependent cytotoxicity to host cells (Garate et al., 2004; Kim et al., 2012). Interestingly, amoebae treated with mannose for 20 cycles exhibited larger vacuoles occupying the most area of the amoebic cytoplasm in comparison with the control group amoebae and glucose-treated amoebae. This implies that exogenous mannose could change the composition of amoebal cytoplasm, or act as a nutrient (Yoo and Jung, 2012).

The MBP gene in *A. culbertsoni* has not been characterized yet but the MBP gene in *A. castellanii* has been (Garate *et al*., 2004; Garate *et al*., 2005; Niyyati et al., 2008). Cloning of MBP gene in *A. castellanii* revealed that it is composed of about 3,620 bp with 5 introns and 6 exons and encodes a protein with 833 amino acids (Garate *et al*., 2004). It runs with an apparent

molecular weight of 130 kDa on gels. We have found that MBP in *A. culbertsoni* ran at 83 kDa on gels similar with the size of predicted 85 kDa MBP in *A. castellanii* (Garate et al, 2004. Finally, DG11, IgM of kappa chains was produced and in the analysis of its localization, when the amoeba moved forward, the MBP was concentrated in the direction of its movement as shown at C1 of the Fig. 4. It implied that MBP concentrated in the movement would be associated with the adhesion of the amoeba to a host cell, leading to amoebial cytotoxicity. Recently, the NfCPB and NfCPB-L proteins of *Naegleria fowleri* were observed on cell membranes, especially on psuedopodia and food-cup structures which were associated with cytotoxicity and phagocytosis (Seong et al., 2017).

In this study, MBP was only purified using a column and a polyclonal and monoclonal antibody reacted with a band of 83 kDa. The antibody will be applied into an immunoscreening, which would help to find a full coding gene from the MBP.

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Fig. 1. Silver stained SDS-PAGE showing the purification of *A. culbertsoni* MBP. PM; pre-stained marker, ly; *A. culbertsoni* lysate, X; blank. An arrow indicates MBP of about 83 kDa MW.

Fig. 2. Antibody titer in mice sera after 5^{th} immunization with MBP. Normal of normal mouse serum was used as a negative control. Numbers indicated immunized mice.

Fig. 3. Selection of hybridomas from 96-well plate and subculture into a 75T culture flask, and limiting dilution to obtain a clone of the highest titer. The absorbance was measured against MBP. PBS and normal mice serum were used as negative controls, and polyclonal serum was used as a positive control. A indicated the changes of absorbance of 2BA9 clones in a 96-well culture plate to a 75T flask. B indicated the changes of limit-diluted DG11 clones of the highest titer like A.

Fig. 4. Western blot of purified MBP with the DG11 monoclonal antibody PM; a pre-stained marker. An arrow indicated about 83 kDa band of the purified MBP.

Fig. 5. Localization of MBP in *A. culbertsoni* trophozoites and cross-reactivity with other *Acanthamoeba* spp. by immunocytochemistry. Arrows indicated *A. culbertsoni* trophozoites by fluorescence microscopy. X1000.

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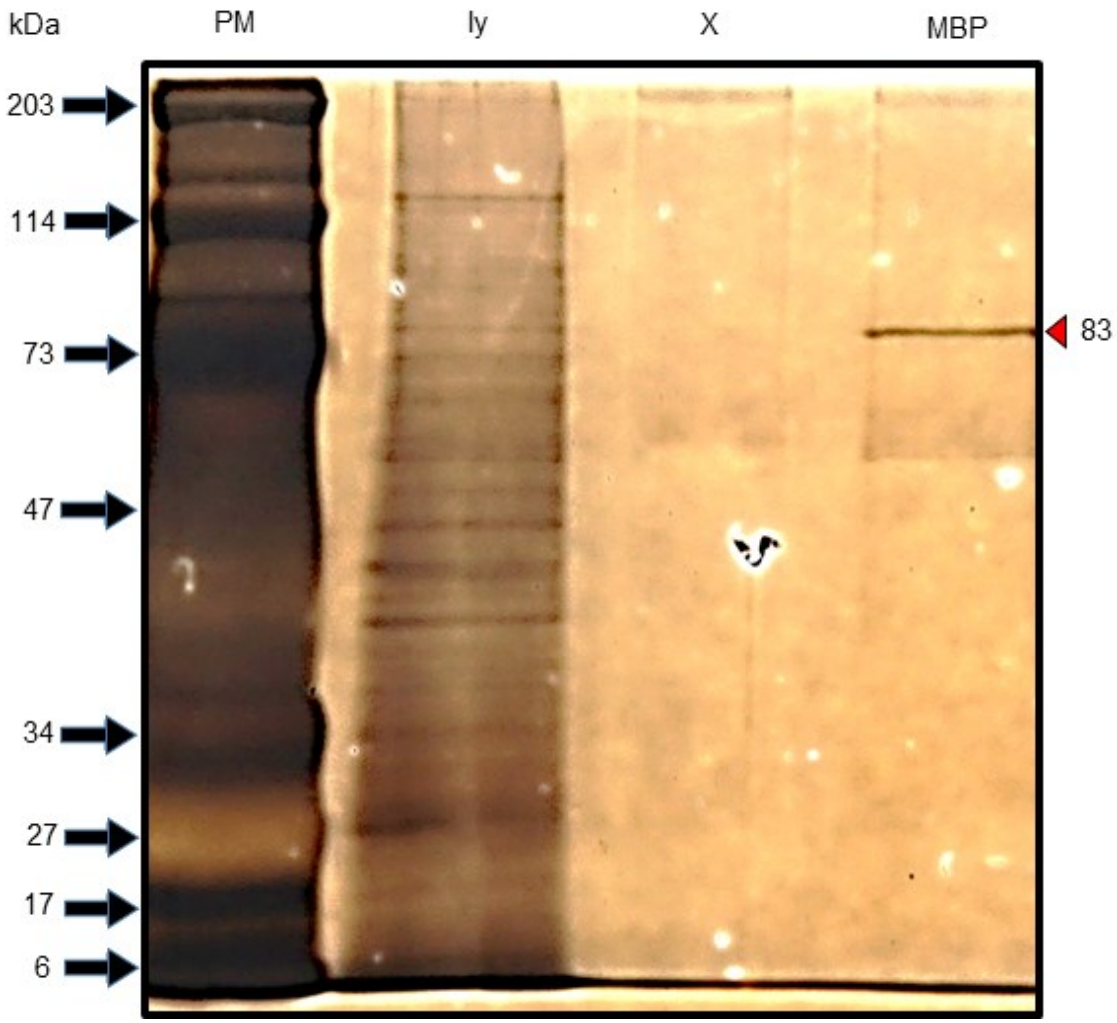

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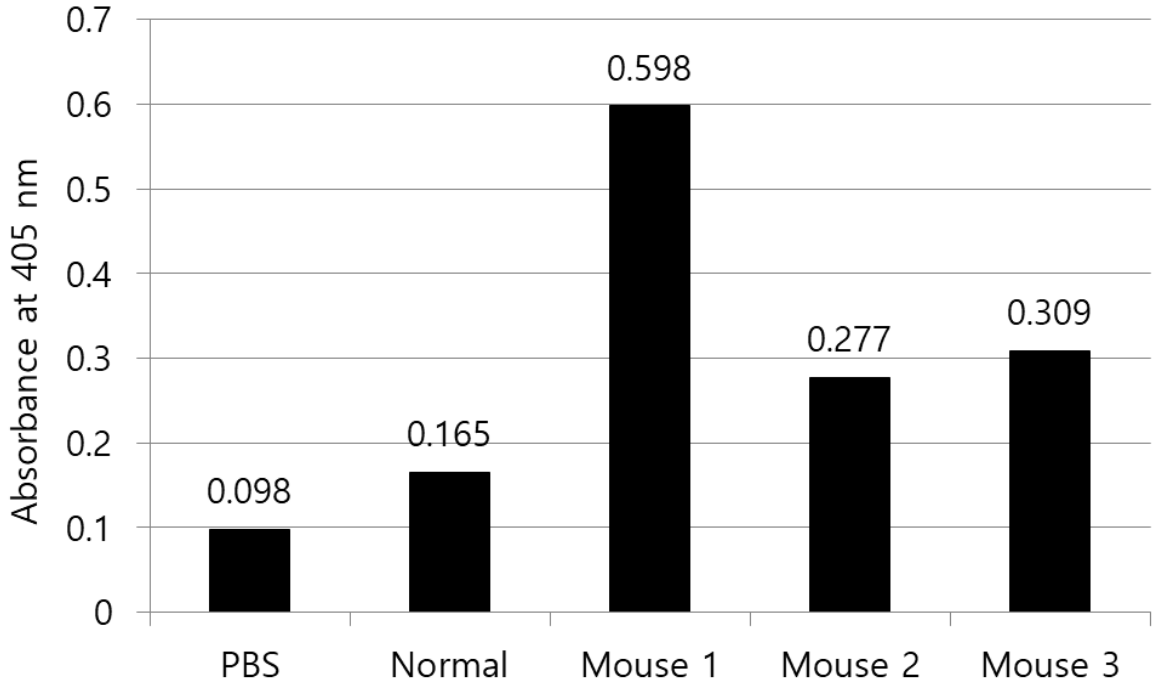
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Fig. 2.

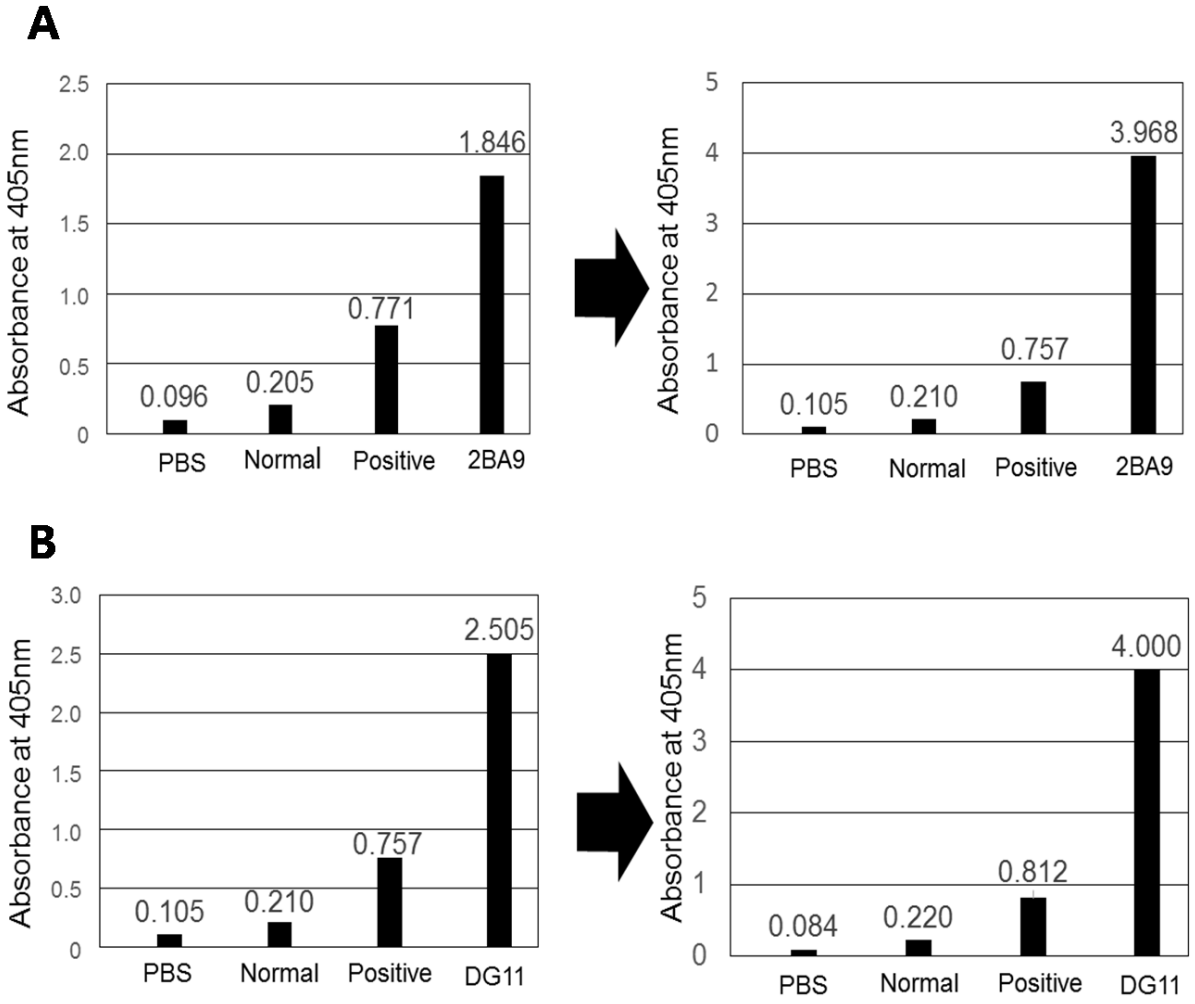
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Fig. 3.

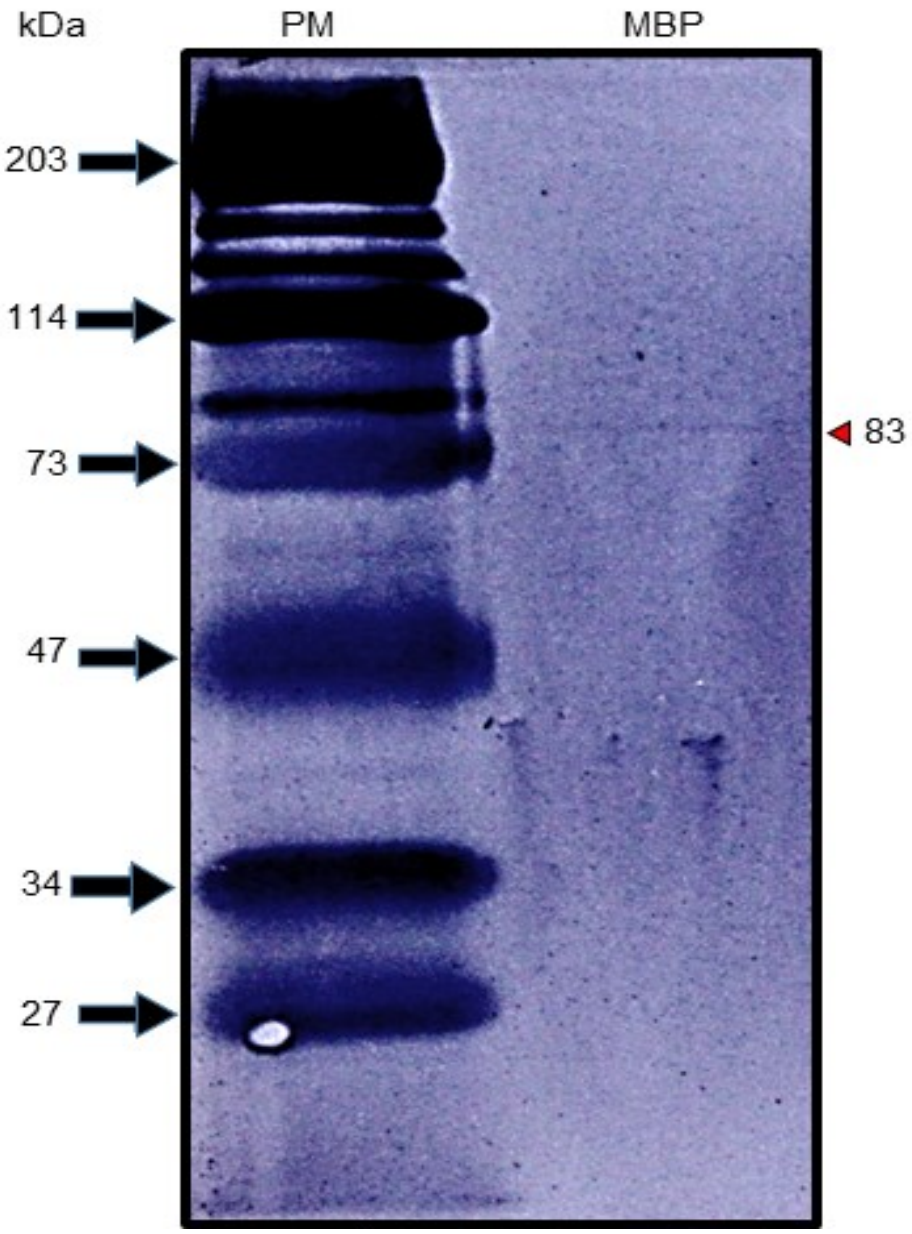

Fig. 4.

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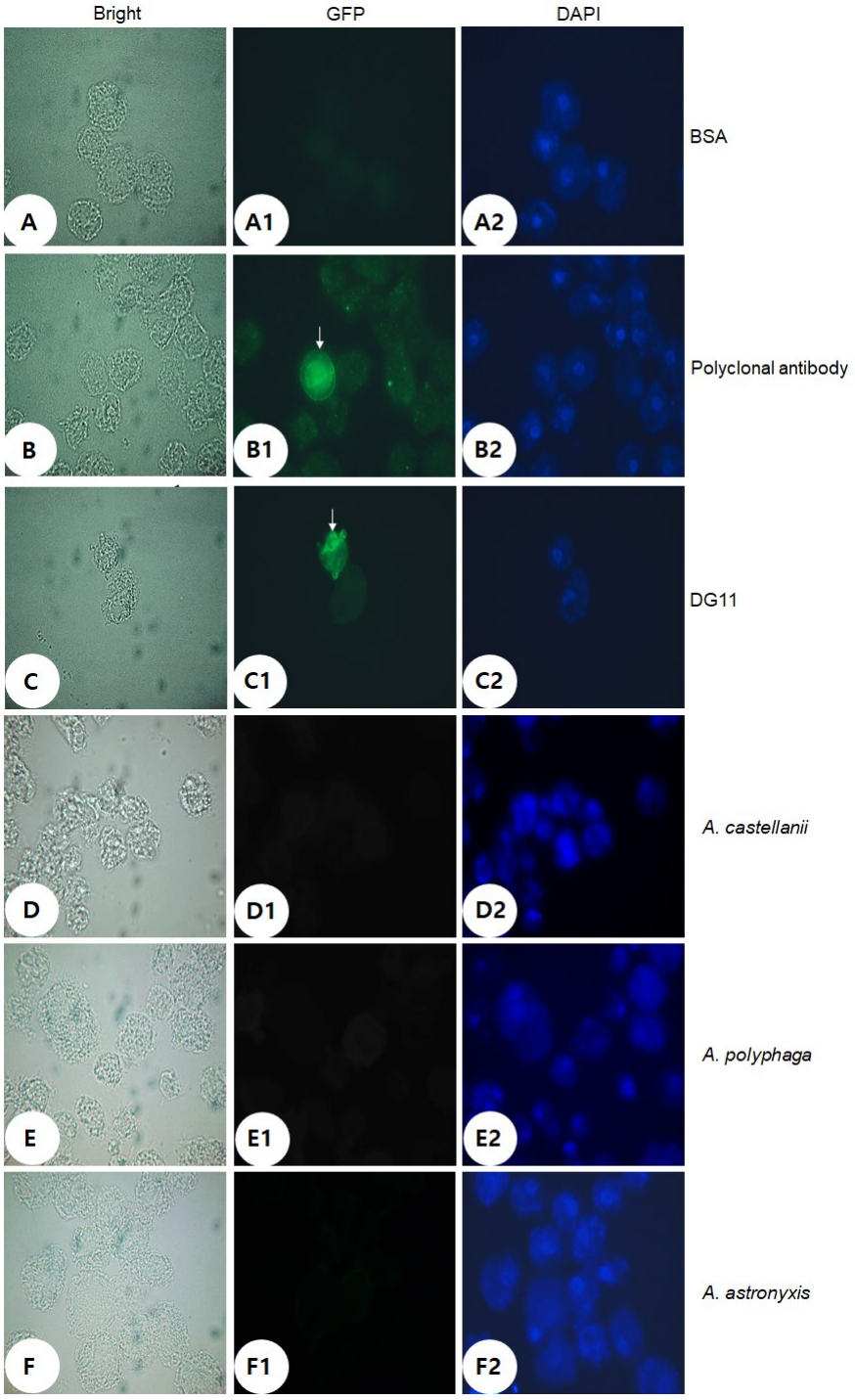
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GFP
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BSA
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DG11
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A. polyphaga
A. astronyxis
A
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D
D1
D2
E
E1
E2
F
F1
F2

4

Fig. 5.